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Phosphodiesterase D is Involved in Bile Resistance in Listeria monocytogenes

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Phosphodiesterase D is Involved in Bile Resistance in *Listeria monocytogenes*

by

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ABSTRACT

Listeria monocytogenes is a deadly foodborne bacterium that is responsible for almost 20% of food-related deaths in the United States. *Listeria monocytogenes* contaminates ready-to-eat products such as cheese, deli meat, and ice cream. Once ingested, it invades the intestinal lining and can enter the bloodstream, causing listeriosis. There is a gap in the knowledge of the pathogenesis of *L. monocytogenes* in how it is able to survive in the gastrointestinal tract in the presence of bile, which has bactericidal properties. Previous studies have suggested that the second messenger cyclic-dimeric-GMP may be involved in the regulation of virulence factors of *Listeria*. This nucleotide is produced by diguanylate cyclases and degraded by phosphodiesterases. The purpose of this study was to determine whether phosphodiesterase D was responsible for bile survival and if oxygen availability influences the impact of this phosphodiesterase. Survival of the wild-type strain (F2365) and the *pdeD* mutant was analyzed in aerobic and anaerobic conditions in neutral and acidic pH with and without 1% bile to mimic locations within the body where bile would be present (i.e. duodenum and gall bladder). Results showed that the *pdeD* mutant was more sensitive to bile in anaerobic and acidic conditions than the wild type. In order to better understand the relationship between PdeD and bile, real-time qPCR was conducted to determine if there were differences in the expression of *bsh* in *pdeD* and F2365. Bsh is the bile salt hydrolase that is used to detoxify bile. Using the 16S gene as an internal control, it was found that there was a slight decrease in expression of *bsh* in *pdeD* than F2365, though this change was not significant. These data suggest that the phosphodiesterase D may be involved in responding to bile-induced damage, but does so independently of the *bsh* expression. The

reduction in bile survival exhibited by this strain suggests that the phosphodiesterase may be responsive to oxidative stress. Further research is needed to determine if the regulation of the *pdeD* is due to exposure to oxidative stress.

Keywords: Listeria monocytogenes, anaerobic environment, phospohdiesterase D, *pdeD*, Bsh, bile

DEDICATION

This honors thesis is dedicated to my parents, who have always supported me in every endeavor, and especially to my grandma, who has always been my biggest cheerleader.

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I want to thank my advisor and mentor, Dr. Janet Donaldson, for believing in me and letting me work with her at the beginning of my undergraduate career. This would not have been possible without you. Thank you for always being a great source of guidance and always answering all of my questions, even when I would frantically call you from the lab. I would also like to thank graduate student Damayanti Chakravarty for teaching me almost all my lab skills and always helping me troubleshoot all of the procedures.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

pdeD	Phosphodiesterase D Gene
PdeD	Phosphodiesterase D Protein
pdeB	Phosphodiesterase B Gene
PdeB	Phosphodiesterase B Protein
pdeC	Phosphodiesterase C Gene
PdeC	Phosphodiesterase C Protein
qPCR	Quantitative Polymerase Chain Reaction
bsh	Bile Salt Hydrolase Gene
Bsh	Bile Salt Hydrolase
Cyclic-dimeric-GMP	Cyclic-dimeric-Guanosine Monophosphate
DgcA, DgcB, DgcC	Diguanylase cyclases A, B, and C
BHI	Brain Heart Infusion
PBS	Phosphate Buffered Saline
Ct Value	Cycle Threshold Values

CHAPTER I: Introduction

Listeria monocytogenes is a gram-positive foodborne facultative anaerobe that primarily affects the immunocompromised, pregnant women, and the elderly. It typically enters the body through contaminated foods, such as ready-to-eat foods, deli meats, cheeses, ice cream, and unwashed produce (1). *Listeria* is able to cross the intestinal lining, blood-brain barrier, and placental barrier (2). This allows *Listeria* to cause meningitis, as well miscarriage or stillbirth. *Listeria monocytogenes* causes listeriosis, which can have a fatality rate of up to 20%, making it one of the most deadly foodborne pathogens (1). In order to effectively treat listeriosis, there needs to be a better understanding of how *L. monocytogenes* is able to survive within the gastrointestinal system, which has varying concentrations of oxygen, bile, and pH (3).

Previous studies have suggested that the nucleotide cyclic-dimeric-Guanosine Monophosphate (cyclic-di-GMP) may be involved in the pathogenesis of *L. monocytogenes* and how it can survive the stressors of the gastrointestinal tract. Cyclic-di-GMP is a second messenger that is produced by diguanylate cyclases (DgcA, DgcB, and DgeC) and degraded by phosphodiesterases (PdeB, PdeC, and PdeD) in *L. monocytogenes*. In other pathogenic bacteria, such as *Escherichia coli*, the cyclic-di-GMP signaling pathway has been shown to contribute to the pathogenicity of the bacteria (4).

In this study, a mutant strain of *Listeria* not containing *pdeD* was compared to the wild-type strain F2365. The goal of this study was to determine if the phosphodiesterase PdeD was required for survival in the presence of bile under aerobic and anaerobic conditions and to determine if the expression of the bile resistance gene *bsh* changes

under the indicated conditions. Our studies found that the *pdeD* mutant was more sensitive to bile under anaerobic acidic conditions and that *pdeD* had a lower expression of *bsh* under anaerobic conditions. This information is important because it indicates that PdeD could be involved in bile survival of *L. monocytogenes* in the acidic and anaerobic environments of the gut.

CHAPTER II: Review of Literature

General Characteristics and Life Cycle of *Listeria*

Listeria monocytogenes is an intracellular foodborne pathogen that causes listeriosis (1). Listeriosis mainly affects immunocompromised individuals and usually manifests as sepsis or meningitis. In pregnant women, *Listeria* can cross the placental barrier and infect the fetus (2). *Listeria* is ubiquitous, meaning that it is found easily in the environment (4). *Listeria monocytogenes* is able to grow at a wide variety of temperatures (1 to 45˚C) and this allows it to contaminate many different types of foods such as produce, deli meats, cheeses, and ice cream (7).

After someone ingests food that is contaminated with *Listeria*, the bacteria enter the gastrointestinal tract. *Listeria monocytogenes* then overcomes antimicrobial stressors of the gut as it passes through the stomach and then the intestines (3). Once in the intestinal tract, *Listeria* invades intestinal epithelial cells using invasion factors InlA or InlB. Once in the cell, it is able to spread to surrounding cells through the formation of listeriapods (5). This method of cell-to-cell spread prevents *L. monocytogenes* from being effectively detected by the immune system, especially one that is weakened.

Environmental Conditions of the Gut

Listeria is able to withstand the many stressors of the human gastrointestinal tract. These include different levels of oxygen, bile, and pH (3). As *Listeria* passes through the gastrointestinal tract, it encounters acidity in the stomach, high concentrations of bile in the small intestine and gallbladder, and varying oxygen levels (14). A decrease in pH is the first stressor of the gastrointestinal tract that *L. monocytogenes* has to be able to withstand. *Listeria* first encounters an acidic environment in the stomach (3). Prior

studies have shown that Listeria has a higher survival in acidic environment when grown under anaerobic conditions (9). The next stressor *L. monocytogenes* encounters is the acidic bile condition of the duodenum. Bile is produced in the liver, stored in the gallbladder, and released in the small intestine during food digestion (3). Bile is composed of cholesterol, bile acids, phospholipids, and water (10). Bile is synthesized from cholesterol in the liver, and then further processed by conjugation with amino acids prior to transportation to the intestine (12). *Listeria* possesses a bile salt hydrolase (Bsh) (3). Bsh is an enzyme produced by microbes in order to withstand bile (10). This enzyme works by catalyzing a reaction that breaks down conjugated bile salts by removing amino acids from the bile salts (11). Studies have shown that the ability of several enteric bacteria to survive in bile can be attributed to Bsh (13).

Previous studies have shown bile to influence other pathogenic bacteria to form biofilms, such as *Salmonella enterica* (6). Some studies have also indicated that bile can affect regulation pathways in certain bacteria, which could also cause a faster cell growth compared to bacteria not exposed to bile (6). In *Listeria*, it has been previously observed that exposure to bile does increase the likelihood of biofilm formation (6).

While different oxygen environments are also encountered within the intestinal tract, these changes do not have large impacts on the growth of *Listeria*. However, the changes in oxygen availability can influence how *Listeria* is able to survive stressors of the gastrointestinal tract, such as different pH levels and bile (9). Previous studies from our lab have shown that oxygen availability has an effect on the bile survival of *L. monocytogenes* (15). These studies suggest that oxygen sensing by *L. monocytogenes* is key to survival within the gastrointestinal tract.

Cyclic-Di-GMP Signaling Pathway

Cyclic-dimeric-Guanosine Monophosphate (cyclic-di-GMP) is a bacterial second messenger that has been found to have a role in the regulation of virulence factors of pathogenic bacteria (4). In this signaling pathway, cyclic-di-GMP is produced by diguanylate cyclases and degraded by phosphodiesterases (4). Specifically, *Listeria* has three diguanylate cyclases (DgcA, DgcB, and DgcC) and three phosphodiesterases (PdeB, PdeC, PdeD) that are a part of this signaling pathway (4).

There are few studies regarding the phosphodiesterases of this specific pathway in *Listeria*. A study by Chen et al. found that deletion of all three phosphodiesterases in *L.* monocytogenes (*pdeB, pdeC,* and *pdeD)* caused an increase in the intracellular concentration of cyclic di-GMP (4). This study suggested that these are the three main phosphodiesterases that break down cyclic-di-GMP. In this previous study, each individual phosphodiesterase (*pdeB*, *pdeC*, and *pdeD*) was restored individually and the motility of *Listeria* in a semi-solid media was measured. Any increases in motility for each specific phosphodiesterase indicated the respective level of activity in the cyclic di-GMP pathway. PdeD specifically had the highest increase in motility, suggesting that it has a major role in the breakdown of cyclic-di-GMP in comparison to PdeB or PdeC (4). Because of that result, we wanted to test if phosphodiesterase D had a part in the bile survival of *Listeria* in different conditions that mimic the gastrointestinal tract. It has been previously shown that bile resistance of *L. monocytogenes* increases under physiologically relevant anaerobic conditions (17). Therefore, we wanted to determine if the changes in the cyclic-di-GMP pathway corresponded to a reduction in bile survival. Though there have been multiple studies on bile survival of *L. monocytogenes*, the exact

mechanism is not known and there are few studies outlining the effects in physiologically relevant anaerobic environments. The goal of this study therefore was to further explore the role of PdeD in the bile survival of *L. monocytogenes* under aerobic and anaerobic conditions. Additionally, variations in pH were also tested. The duodenum contains bile at a pH of approximately 5.5, while the gallbladder contains bile at a pH of approximately 7.5. The acidic conditions are known to increase the toxicity of bile.

CHAPTER III: Methodology

Bacterial Strains, Cell Culture, and Treatments

Listeria monocytogenes strain F2365 is a 4b serotype that has been previously sequenced (7). This strain was isolated from a Jalisco Cheese Outbreak of *Listeria* in 1985 (8). The *pdeD* mutant was previously generated in the F2365 background in the Donaldson laboratory and was verified to be correct via PCR and gene sequencing.

As shown in Figure 3.1, the *pdeD* mutant and wild-type strain F2365 were tested under multiple conditions. Brain Heart Infusion (BHI) media was used for all culture conditions tested. All cultures were incubated at 37˚C. The bile solution contained BHI media, 1% bile porcine (Sigma), and 0.01% methanol. For treatments requiring acidic media, hydrochloric acid was added to BHI to a pH of 5.5 as measured using a Fisher Scientific accumet AE150 pH probe.

Aerobic conditions were achieved by incubating cultures at standard atmospheric conditions. Anaerobic conditions were achieved using a Coy Anaerobic Chamber with 5% H_2 and balanced with N_2. Plated samples from anaerobic conditions were incubated in a sealed box with anaerobic pouches.

Bacterial Growth Curves

Overnight cultures were grown in BHI at 37˚C under aerobic conditions. To achieve cultures in exponential phase, 100µL aliquots of overnight cultures were transferred to 5mL of fresh BHI and incubated in a shaking incubator at 37˚C. Cultures were grown to an OD_{600} of 0.3 to 0.5; the OD_{600} of the cultures was measured using a NanoDrop 100 Spectrophotometer. Each sample was split into two 2 mL aliquots and centrifuged for 4 minutes at 7500 x *g*. After decanting the supernatant, the pellets were

resuspended in the corresponding treatment (0% or 1% bile, pH of 7 or 5) in either aerobic or anaerobic conditions, as shown in Figure 3.1. The samples were then incubated at 37˚C and aliquots were diluted and plated after 0, 1, 3, 5, and 7 hrs. After incubating serial dilutions for 24 hrs at 37˚C in either aerobic or anaerobic conditions, colonies were counted, and the percent survival was calculated in relation to 0 hr. A minimum of three independent replicates was performed. Statistical analysis was conducted using a two-tailed T test.

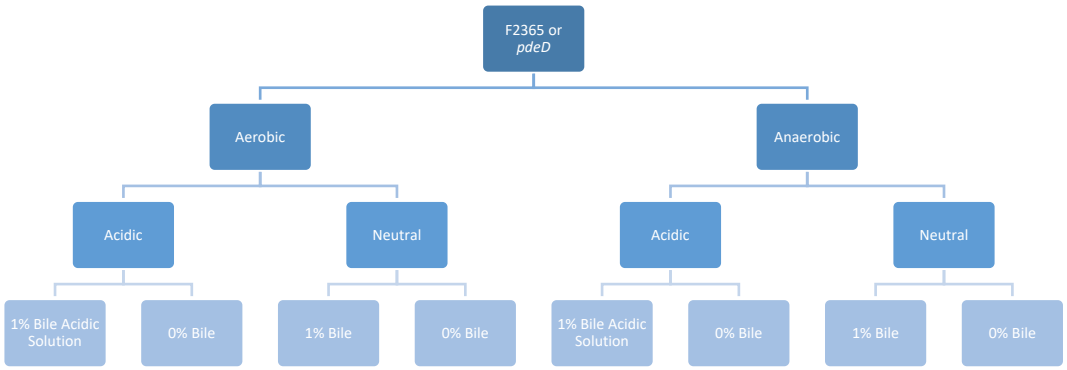

Figure 3.1. Conditions tested for F2365 and pdeD.

This diagram illustrates the various conditions under which the growth curves for *pdeD* and F2365 were conducted.

RNA Isolation and qPCR

RNA was isolated from F2365 and *pdeD* mutant cultures grown to midlog and treated for 1 hr with either 0% or 1% porcine bile (Sigma) under aerobic and anaerobic conditions. Each sample was washed with PBS and treated with Bacterial RNAProtect (Qiagen) before isolation. The RNAeasy Plus Mini kit and QIAshredder kits were used

for the RNA isolation (Qiagen). The cells were then lysed using 0.5mm zirconia beads and a bead mill homogenizer two times for 2 min with a 1 min rest period at 5 meters per second. The RNA was then converted to cDNA using an Applied Biosystems High Capacity cDNA Reverse Transcription Kit. The cDNA was then quantified using a Qubit 4.0 Fluorometer and an Invitrogen Qubit dsDNA BR Assay Kit. Then qPCR was conducted using 25 ng/µL of each sample, TaqMan Gene Expression master mix containing *bsh* primers, and an internal control of the 16S gene, as shown in Table 3.1. The cycle threshold values (Ct values) were determined using a Step One Real Time PCR System.

Primers	**Sequence (5'-3')**
16S	
Forward	GTG GAG CAT GTG GTT TAA TTC G
Reverse	ACC CAA CAT CTC ACG ACA C
Probe	/56-FAM/CCA CCT GTC /ZEN/ACT TTG TCC CCG AA/3IABkFQ/
bsh	
Forward	TCC ATT TTC GCA AGG TAG AGG
Reverse	TCC TGA GAA ATT GAG TCC TGC
Probe	/56-FAM/ACA ACG GGT /ZEN/AGT TTT CCA TCA CAG CA/3IABkFQ/

Table 3.1. Primers and qPCR probes used for real-time PCR of F2365 and *pdeD*.

This table lists the forward and reverse primer sequences and probes for *16S* and *bsh*.

CHAPTER IV: Results

Anaerobic Conditions Increased Survival of F2365 in Bile.

The wild-type strain F2365 had a slight, yet significant, decrease in survival when exposed to bile under aerobic neutral conditions ($p < 0.02$; Figure 4.1). In anaerobic conditions, the bile survival did not significantly change (Figure 4.2).

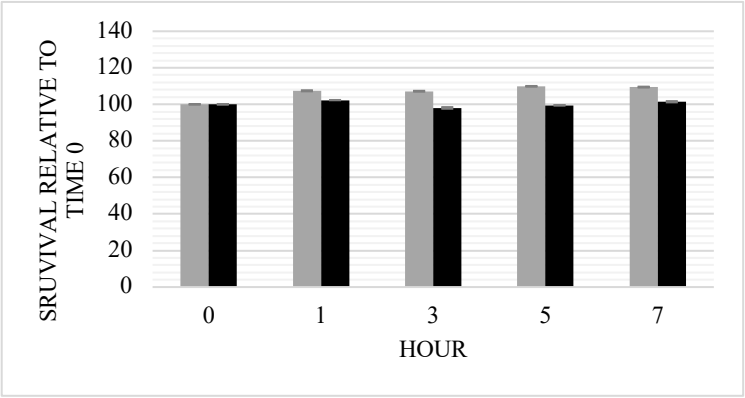

Figure 4.1. Bile Survival of F2365 in Aerobic Conditions at a pH of 7.5

Samples were grown to midlog aerobically and then transferred to media containing 1% bile or 0% bile and plated at 0, 1, 3, 5, and 7 hrs. The black bars represent the samples grown to midlog in 1% bile media and the gray bars represent the samples grown in 0% bile media. Survival was determined in response to growth at time 0 hr. Data represent the mean of survivals conducted in triplicate.

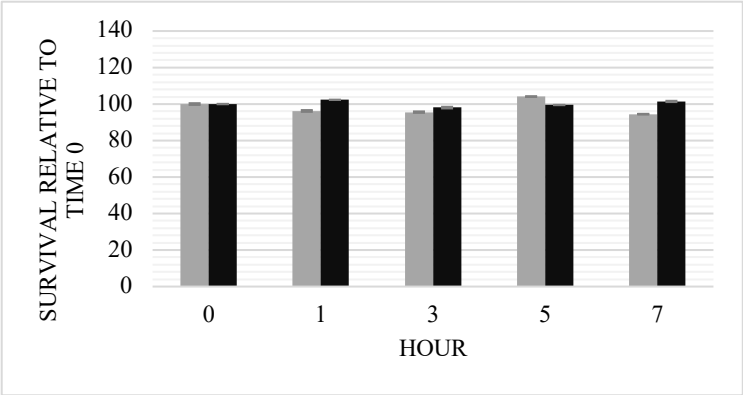

Figure 4.2. Bile Survival of F2365 in Anaerobic Conditions at a pH of 7.5

Samples were grown to midlog in an anaerobic chamber and then transferred to media containing 1% bile or 0% bile and plated at 0, 1, 3, 5, and 7 hrs. The black bars represent the samples grown to midlog in 1% bile media and the gray bars represent the samples grown in 0% bile media. Survival was determined in response to growth at time 0 hr. Data represent the mean of survivals conducted in triplicate.

Acidic Conditions Decreased Survival of F2365 in Bile.

To mimic the conditions of the duodenum, *L. monocytogenes* wild-type strain F2365 was exposed to bile at a pH of 5.5. In both aerobic and anaerobic conditions, the wild-type strain had a decrease in survival in bile in comparison to controls (Figure 4.3, Figure 4.4). In anaerobic conditions, the difference between the bile conditions at a pH of 7.5 (Figure 4.2) and pH 5.5 (Figure 4.4) was found to be statistically significant ($p < 0.02$). In an acidic aerobic environment (Figure 4.3), the difference between survival of F2365 in bile and no bile was also found to be statistically significant ($p < 0.02$).

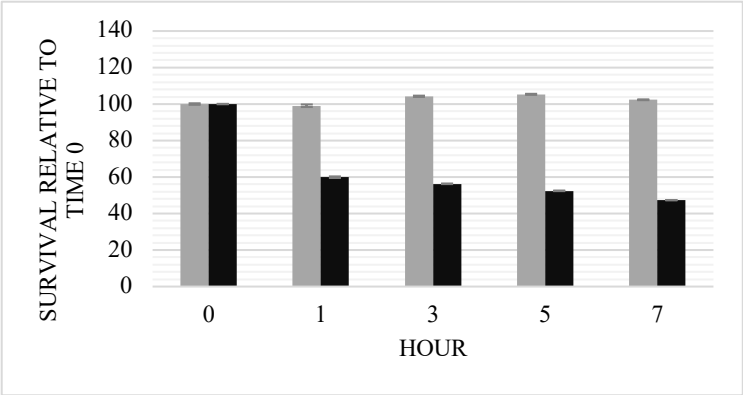

Figure 4.3. Survival of F2365 in the Presence of Bile Under Aerobic Conditions at a pH of 5.5

Samples were grown to midlog aerobically and then transferred to acidic media containing 1% bile or 0% bile and plated at 0, 1, 3, 5, and 7 hrs. The black bars represent the samples grown to midlog in 1% bile media and the gray bars represent the samples grown in 0% bile media. Survival was determined in response to growth at time 0 hr. Data represent the mean of survivals conducted in triplicate.

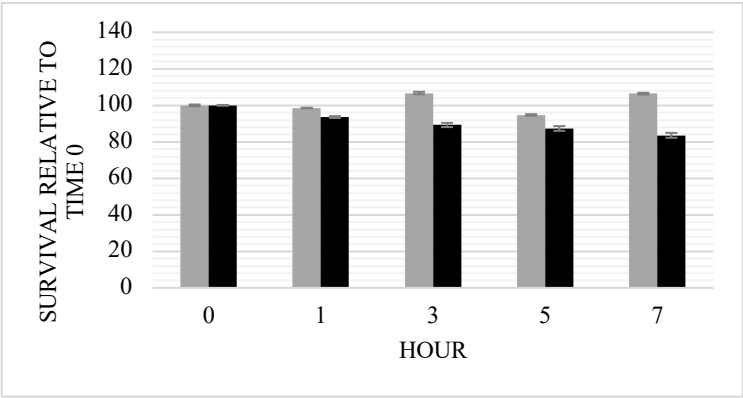

Figure 4.4. Bile Survival of F2365 in Anaerobic Conditions at a pH of 5.5

Samples were grown to midlog in an anaerobic chamber and then transferred to acidic media containing 1% bile or 0% bile and plated at 0, 1, 3, 5, and 7 hrs. The black bars represent the samples grown to midlog in 1% bile media and the gray bars represent the samples grown in 0% bile media. Survival was determined in response to growth at time 0 hr. Data represent the mean of survivals conducted in triplicate.

Acidic Conditions Decreased Survival of *pdeD* in Bile.

In neutral environments under both aerobic and anaerobic conditions, bile did not impact the survival of the *pdeD* mutant (Figure 4.5, Figure 4.6). However, as shown in Figure 4.7, there was a significant decrease in bile survival of *pdeD* from hour 1 to hour 7 in aerobic acidic environment ($p < 0.02$). In an anaerobic acidic environment, the bile survival of *pdeD* also decreased significantly (Figure 4.8; $p < 0.02$). This decrease was also significantly different than the decrease observed with the wild-type strain (Figure 4.4)

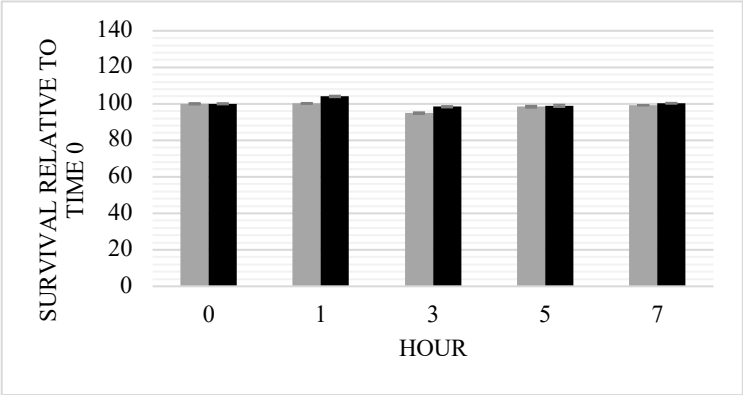

Figure 4.5. Bile Suvival of the pdeD Mutant in Aerobic Conditions at a pH of 7.5

Samples were grown to midlog aerobically and then transferred to media containing 1% bile or 0% bile and plated at 0, 1, 3, 5, and 7 hrs. The black bars represent the samples grown to midlog in 1% bile media and the gray bars represent the samples grown in 0% bile media. Survival was determined in response to growth at time 0 hr. Data represent the mean of survivals conducted in triplicate.

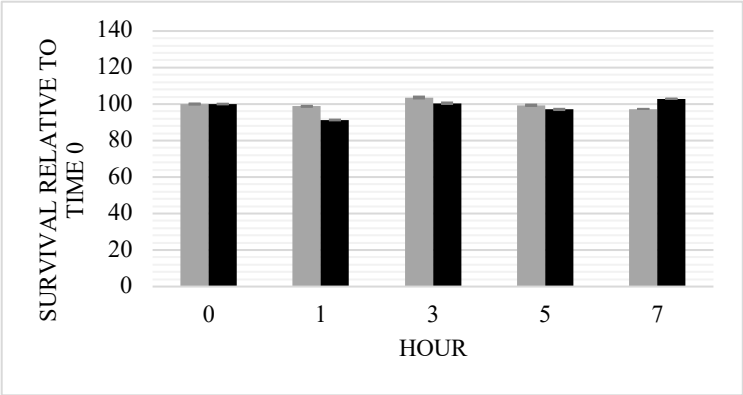

Figure 4.6. Bile Survival of the pdeD Mutant Under Anaerobic Conditions at a pH of 7.5

Samples were grown to midlog in an anaerobic chamber and then transferred to media containing 1% bile or 0% bile and plated at 0, 1, 3, 5, and 7 hrs. The black bars represent the samples grown to midlog in 1% bile media and the gray bars represent the samples grown in 0% bile media. Survival was determined in response to growth at time 0 hr. Data represent the mean of survivals conducted in triplicate.

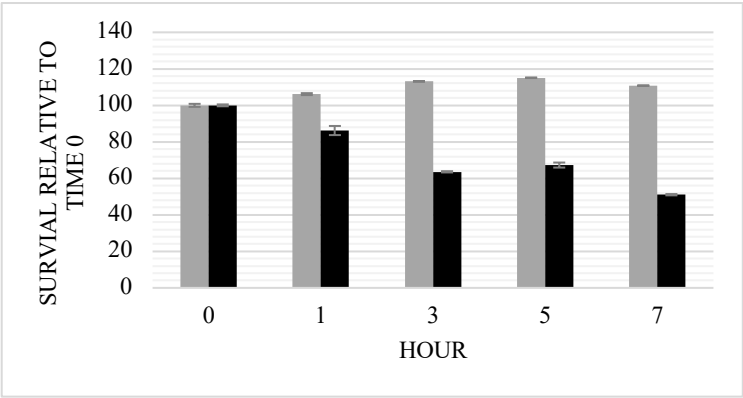

Figure 4.7. Bile Survival of the pdeD Mutant Under Aerobic Conditions at a pH of 5.5

Samples were grown to midlog aerobically and then transferred to acidic media containing 1% bile or 0% bile and plated at 0, 1, 3, 5, and 7 hrs. The black bars represent the samples grown to midlog in 1% bile media and the gray bars represent the samples grown in 0% bile media. Survival was determined in response to growth at time 0 hr. Data represent the mean of survivals conducted in triplicate.

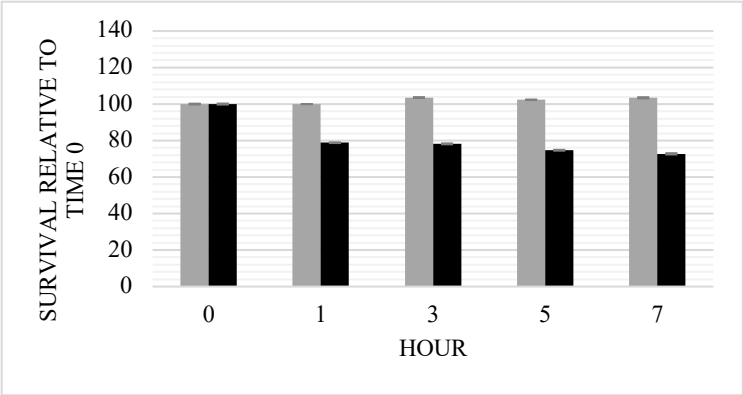

Figure 4.8. Bile Survival of the pdeD Mutant in Anaerobic Conditions at a pH of 5.5

Samples were grown to midlog in an anaerobic chamber and then transferred to acidic media containing 1% bile or 0% bile and plated at 0, 1, 3, 5, and 7 hrs. The black bars represent the samples grown to midlog in 1% bile media and the gray bars represent the samples grown in 0% bile media. Survival was determined in response to growth at time 0 hr. Data represent the mean of survivals conducted in triplicate.

The *pdeD* Mutant and F2365 Did Not Decrease Expression of Bsh in Anaerobic Conditions.

RNA was isolated one hour after midlog for the anaerobic conditions shown in Table 4.1. The average Ct values from the qPCR for 16S and *bsh* (Table 4.1) were used to calculate the fold change for F2365 and *pdeD* mutant (Figure 4.9), using 16S as an internal control (Table 4.2). F2365 had a fold change of 1.04, whereas *pdeD* had a fold change of 0.34. Neither of these are biologically significant changes in expression.

Sample	Average cDNA Concentration (ng/µL)
F2365 0% Bile	5.18
F2365 1% Bile	8.20
PdeD 0% Bile	5.61
PdeD 1% Bile	7.61

Table 4.1. Average cDNA concentration of each sample in anaerobic conditions.

The cDNA concentration was measured using a Qubit 4.0 Fluorometer and an Invitrogen dsDNA BR Assay kit. Data represents the mean of samples conducted in triplicate.

0% Bile PdeD	average Ct	standard deviation	ΔCt
16S	22.26	4.84	1.42
Bsh	23.68	4.70	
1% Bile PdeD			
16S	21.67	1.44	2.98
Bsh	24.65	0.75	
0% Bile F2365			
16S	15.9775	3.00	6.33
Bsh	22.30625	3.40	
1% Bile F2365			
16S	21.555	3.83	6.27
Bsh	27.825	2.46	

Table 4.2. Average Ct values of *bsh* in the *pdeD* and F2365 strains in anaerobic conditions.

Ct values were obtained from qPCR of cDNA from PdeD and F2365, with or without bile in anaerobic conditions. The difference between the average Ct values for each probe was defined as ΔCt for each strain and condition. Data represent the mean of samples conducted in triplicate.

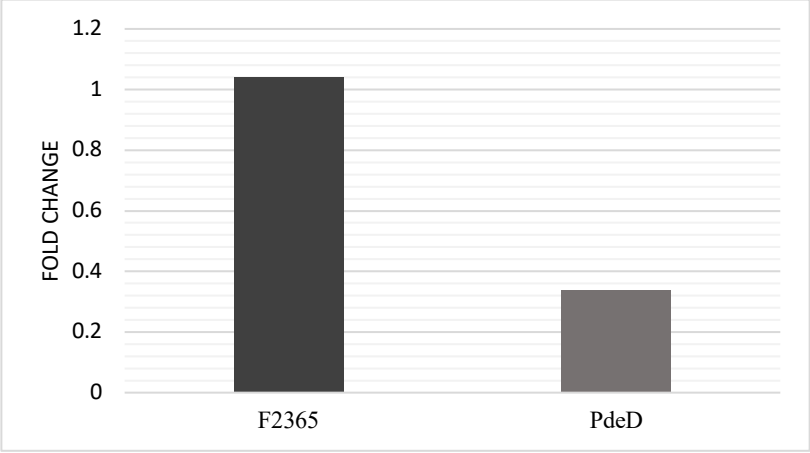

Figure 4.9. The expression of Bsh in F2365 and pdeD mutant in anaerobic conditions.

Fold change was calculated using the Ct values from qPCR using a Bsh probe and a 16S probe. Data represents the mean of samples conducted in triplicate.

CHAPTER V: Discussion

Listeria monocytogenes is one of the leading causes of foodborne related illnesses in the United States, causing 2500 cases annually and has a fatality rate of up to 20% (8). *Listeria* infection causes listeriosis, which can be deadly in immunocompromised groups. It is important to understand the pathogenesis of these bacteria, but there is not enough known about *Listeria* in that regard. The purpose of this study was to help close the gap in the knowledge of *Listeria* and how it is able to survive in the human gastrointestinal tract. The gut has many lines of defense in place to prevent infection, such as varying levels of acidity, bile, and oxygen (3). The cyclic-di-GMP signaling pathway may allow *Listeria* to withstand the stressors of the gut. Previous studies have shown that the phosphodiesterase D (PdeD) may have a role in the pathogenicity of *Listeria* (4). Another phosphodiesterase, PdeC, has been shown to have a role in the cyclic di-GMP pathway regarding exopolysaccarides expressed by *Listeria*, but the role of PdeD in this pathway has not been specifically studied (4).

In order to understand if phosphodiesterase D has a role in bile survival of *Listeria*, a *pdeD* mutant was used as a comparison to *Listeria* strain F2365. A bacterial growth curve analysis was conducted for both *pdeD* and F2365 in conditions with or without bile, neutral or acidic, and aerobic or anaerobic. While there were not many significant differences in the conditions, the bile survival of *pdeD* decreased in the anaerobic acidic conditions significantly in comparison to the wild-type strain. High cyclic di-GMP levels have been associated with lower levels of virulence factors of microbes (16). It could be likely that the lack of *pdeD* prevented the full breakdown of cyclic-di-GMP in the pathway, which could cause it to have lower bile survival under

anaerobic and acidic conditions encountered in the duodenum. This may suggest that *pdeD* has a role in the bile survival of *Listeria* in these conditions.

Using the data from the growth curve analysis, it was decided to isolate RNA under anaerobic neutral conditions with and without bile from *pdeD* and F2365. After the RNA Isolation, the samples were converted to cDNA and quantified. After cDNA quantification, real-time qPCR was conducted using 16S and Bsh probes. The Ct values for 16S were used as an internal control to calculate the fold change of Bsh in *pdeD* and F2365. While not statistically significant, the fold change for *pdeD* was slightly lower than F2365. The mutant strain having a lower level of *bsh* than F2365 was to be expected because the lack of phosphodiesterase D in the mutant strain probably interrupted the cyclic di-GMP regulation pathway. This could indicate that expresion of *bsh* in *Listeria* may rely on this pathway, but more research needs to be conducted in this area to support this.

While this study provides more information on how *Listeria* is able to combat the stressors of the gut, more research in this area is necessary to fully understand the pathogenesis of these bacteria. Future directions include continuing RNA isolations and real-time qPCR for *pdeD* mutant and F2365 but under anaerobic acidic conditions or even using different probes for the qPCR. Because of the large gap in the knowledge of phosphodiesterases and ther roles in *Listeria*, *pdeB* and *pdeC* could also be studied independently to see how all of the currently identified phosphodiesterases affect the bile survival of *Listeria*. Further research on the pathogenesis of *Listeria* can help improve to improve treatments for listeriosis and even help to reduce outbreaks.

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